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(54) Title: COMPOSITIONS AND METHODS FOR INDUCING CELL DEATH

(57) Abstract

A method is provided for inducing cell death comprising administering to said cell a composition comprising tempo or a functional derivative of tempo.

TITLE OF THE INVENTION

Compositions and Methods for Inducing Cell Death

Field of the invention

5   This invention relates to compositions and
methods for inducing cell death, and treatment of
diseases and conditions where cell death is
beneficial.

Introduction

10   A cellular antioxidant defense system composed of
enzymes such as catalase, superoxide dismutase,
glutathione peroxidase, and glutathione protects cells
against toxic oxygen metabolites. Exogenously added
free radical scavengers have also been shown to
15   alleviate the deleterious effects of oxygen free
radicals (van Asbeck, B. S. et al., 1985, Science 227,
756-759; Halliwell, B. 1989, Free Radical Biol. Med.
7, 645-651; Myers, M. L. et al., 1985, Circulation 72,
915-921; Quintanilha, A. T. and Packer, L., 1977,
20   Proc. Natl. Acad. Sci. U.S.A. 74, 570-574). Nitroxide
compounds, including tempol and tempo (Fig. 1), are
low molecular weight, membrane permeable, stable free
radicals that are electron paramagnetic resonance
detectable (Berliner, L. J. , 1976 Spin Labeling:
25   Theory and Application, Academic Press, New York) and
have been used classically as probes for biophysical
and biochemical processes; they have been used as
paramagnetic contrast agents in NMR imaging (Bennett,
H. F. et al., 1987, Magn. Reson. Med. 4, 93-111;
30   Bennett, H. F. et al., 1987, Invest. Radiol. 22, 502-
507), as probes for membrane structure (Berliner, L.
J. 1979, Spin Labeling II: Theory and Applications,
Academic Press, New York), and as sensors of oxygen in
biological systems (Strzalka, K. et al., 1990, Arch.

Biochem. Biophys 281: 312-318). However, over the
past few years, novel applications of nitroxide have
been demonstrated. Nitroxides have been shown to
possess antioxidant activity and protect cells against
5   a variety of agents that impose oxidative stress,
including superoxide, hydrogen peroxide, and ionizing
radiation (Mitchell, J. B. *et al.* 1990, *Biochemistry*
29, 2802-2807; Smuni, A. *et al.*, 1990, *Adv. Exp. Med.*
Biol. 264, 85-92; Samuni, A. *et al.*, 1990, *Free*
10   *Radical Res. Commun.* 9, 241-249; Mitchell, J. B. *et*
al., 1991, *Arch. Biochem. Biophys.* 289, 62-70; Samuni,
A. *et al.*, 1991, *Biochemistry* 30, 555-561; Hahn, S. M.
et al., 1992, *Cancer Res.* 52, 1750-1753; Hahn, S. M.
et al., 1992, *Radiat. Res.* 132, 87-93; Hahn, S. M. *et*
15   *al.*, 1995, *Can. J. Physiol. Pharmacol.* 73, 399-403;
Samuni, A. *et al.*, 1991, *J. Clin. Invest.* 87, 1526-
1530; Gelvan, D. *et al.*, 1991, *Proc. Natl. Acad. Sci.*
U.S.A. 88, 4680-4684; Goffman, T. *et al.*, 1992, *Int.*
J. Radiat. Oncol. Biol. Phys. 22, 803-806). A variety
20   of chemical mechanisms have been proposed to account
for nitroxide antioxidant activity, including
superoxide dismutase mimic activity (Samuni, A. *et*
al., 1988, *J. Biol. Chem.* 263, 17921-17924), oxidation
of reduced metals that would otherwise catalyze the
25   formation of hydroxyl radicals (Krishna, M. C. *et al.*,
1996, *J. Biol. Chem.* 271, 26018-26025), radical-
radical interactions (Mitchell *et al.*, 1990, *supra*).
Although significant research has been conducted at
the whole cell level and in animals with nitroxides,
30   little is known at the molecular level of how this
novel class of antioxidants affects signal
transduction pathways.
        Members of the mitogen-activated protein kinase
(MAPK) family, including extracellular signal-
35   regulated kinase (ERKs) (p42/44 MAPKs), the stress-

activated protein kinases (SAPKs) (also called c-Jun NH2-terminal kinases (p46/54 JNKs/SAPK1)), and p38 MAPK (also termed reactivating kinase (p38RK)), are activated in response to a variety of cellular

5   stresses, such as changes in osmolarity and metabolism, DNA damage, heat shock, ischemia, UV radiation, ionizing radiation, or inflammatory cytokines (Cuenda, A. et al, 1995, *FEBS Lett.* 364, 229-233; Beyaert, R. t al., 1996, *EMBO J.* 15, 1914-

10 1923; Bogoyevitch, M. A. et al., 1996, *Circ. Res.* 79, 162-173; Verheij, C. et al., 1996, *Nature* 380, 75-79; Mendelson, K. G. et al., 1996, *Proc. Natl. Acad. Sci. U.S.A.* 93, 12908-12913; Rosette, C. and Karin, M. 1996, *Science* 274, 1194-1197; Kasid, U. et al., 1996,

15 *Nature* 382, 813-816; Wu, J. et al., 1994, in <u>Insulin Action, Effects on Gene Expression and Regulation and Glucose Transport</u> (Draznin, B. and Le Roith, D., eds) pp. 151-177, Humana Press, Totowa, NJ; Xia, Z. et al, 1995, *Science* 270, 1326-1331; Kyriakis, J. M., and

20 Avruch, J. 12996, *J. Biol. Chem.* 271, 24313-24316; Devary, Y. et al., 1992, *Cell* 71, 1081-1091; Derijard, B. et al., 1995, *Science* 267, 682-685; Sanchez, I. et al., 1994, *Nature* 372, 794-798; Hannun, Y., 1994, *J. Biol. Chem.* 269, 3125-3128; Kharbanda, S. et al.,

25 1995, *Nature* 376, 785-788; Johnson, N. L. et al., 1996, *J. Biol. Chem.* 271, 3229-3237; Hibi, M. et al., 1993, *Genes Dev.* 7, 2135-2148; Kyriakis, J. M. et al., 1994, *Nature* 369, 156-160; Minden, A. et al., 1994, *Science* 266, 1719-1723; Pombo, C. M. et al., 1994, *J.*

30 *Biol. Chem.* 269, 26546-26551; Suy, S. et al., 1997, *Oncogene* 15, 53-61; Westwick, J. K. et al., 1995, *J. Biol. Chem.* 270, 22689-22692). In many of these instances, free radicals and derivatives play an important role in initiating a cellular signal

transduction response (Lander, H. M., 1997, *FASEB J.*
11, 118-124). Unlike the ERK signaling pathway, which
primarily promotes growth and proliferation/survival,
the SAPK and p38 MAPK pathways result in growth arrest
5   and apoptotic or necrotic cell death. Because
nitroxides protect against diverse oxidative insults
and may have utility in clinical biomedical research,
we have investigated the effects of tempol and tempo
on MAPK signal transduction pathways in an attempt to
10 better understand their mechanism of action. Evidence
presented here demonstrates that tempol and tempo
stimulate distinct pathways of the MAPK signaling
cascade. Tempol stimulated the ERK activity and was
noncytotoxic, whereas tempo induced ceramide
15 generation, SAPK/JNK activation, and apoptotic death
of MDA-MB231 human breast cancer cells. The cytotoxic
effect of tempo was also observed in other cancer cell
types, PCI-04A laryngeal squamous carcinoma cells,
androgen-independent (DU145, PC-3) and androgen-
20 dependent (LNCaP) prostate cancer cell lines. Tempo
caused activation of caspase-3, a protease known to
cause apoptosis and chromatin fragmentation as
evidenced by electron microscopy of LNCap cells. In
addition, tempo-treatment caused tumor growth control
25 of MDA-MB231 breast tumor xenografts in athymic mice,
suggesting a therapeutic application of tempo or any
of its derivatives or formulations.

SUMMARY OF THE INVENTION

30   This invention describes tempo for use in
inducing cell death. tempo has been used previously
to protect cells from oxidative damage. The cytotoxic
effect of tempo described in this application is both
both novel and unexpected.

        Therefore, one object of the present invention is
to provide a method for inducing cell death by
introducing to said cell, tissue, or tumor mass a
composition containing tempo or a functional

5   derivative of tempo such that cell death is induced.
        It is another object of the present invention to
provide a composition comprising tempo for inducing
cell death and as a therapeutic composition alone or
in combination with other anticancer agents for the

10 treatment of cancer.
        It is yet another object of the present invention
to provide a method for the treatment of cancer by
administering to a patient a composition comprising
tempo as described above, for example, a liposomal

15 formulation of tempo.

        Brief Description of the Drawings
        These and other features, aspects, and advantages
of the present invention will become better understood

20 with reference to the following description, appended
claims, and accompanying drawings where:
        Figure 1. is a schematic representation of the
chemical structures of nitroxide compounds tempol (4-
hydroxy-tempo) and tempo.

25   Figure 2. Effects of tempol and tempo on protein
tyrosine phosphorylation. Subconfluent cultures were
grown in serum-free medium overnight, followed by
treatment with tempol (10 mM) or tempo (10 mM) for
indicated times and lysis. Normalized protein

30 contents (1 mg) were immunoprecipitated with agarose-
conjugated anti-PY MAb, and then immunoblotted with
anti-PY MAb. Data shown is representative of two
independent experiments. UT, untreated cells grown
overnight in serum-free medium.

Figure 3. Tempol and tempo stimulate tyrosine
phosphorylation and enzymatic activity of Raf-1
protein kinase in vivo. A, Cells were grown in serum-
free medium overnight and treated with tempol (10 mM)
5   or tempo (10 mM) for 15 min and lysed. Normalized
protein contents (1 mg) were immunoprecipitated (IP)
with agarose-conjugated anti-Raf-1 polyclonal
antibody, followed by immunoblotting (IB) with anti-PY
Mab (top). The same blot was stripped and reprobed
10 with anti-Raf-1 Mab (bottom). Data shown is
representative from 2-3 independent experiments. UT,
untreated cells grown in serum-free medium, and
treated with 1% ethanol, B, Raf-1 protein kinase
activity was measured either by a kinase cascade A
15 "read-out" assay (top), or using the Syntide 2
phosphorylation assay (bottom). Cells were grown in
serum-free medium overnight, followed by treatment
with tempol (10 mM) or tempo (10 mM) for indicated
times and lysis. WCLs (1 mg) were immunoprecipitated
20 with agarose-conjugated anti-Raf-1 antibody. For the
coupled-kinase cascade reaction, Raf-1 immune-
complexes were first incubated at 30°C for 30 min with
5 nmol of $[\gamma^{32}P]$-ATP, inactive MAPK kinase (0.4 ug),
and inactive MAPK (1 ug) in 40 ml kinase reaction
25 buffer. MBP (20 ug) was then added to the first
reaction mixture (8 ul) and reaction continued at 30°C
for 10 min in 30 ul kinase reaction buffer. MBP
phosphorylation was quantified using a filter binding
assay as described in materials and methods. For the
30 Syntide 2 phosphorylation assay, Raf-1 immune-
complexes were incubated at 30°C for 20 min with 5
nmol of $[\gamma^{32}P]$-ATP and 5 ug of Syntide-2 in 40 ul
kinase reaction buffer, and Syntide-2 phosphorylation
was quantified using a filter binding assay. Data
35 shown are mean ± standard deviation (s.d.) from 2-3

independent experiments. Control, cells were grown
overnight in serum-free medium and treated with 1 %
ethanol for 1 h (top) or 2 h (bottom).

Figure 4. Tempol stimulates ERK1 activity.

5   Cells were grown in serum-free medium overnight,
treated with tempol (10 mM) or tempo (10 mM) for 2 h
and lysed. WCL (1 mg) were immunoprecipitated with
agarose-conjugated anti-ERK1 antibody, and in vitro
MBP phosphorylation assay was performed as described

10 in experimental procedures. The incorporation of γ^{32}P
into MBP was determined in a filter binding assay (A).
In other independent experiments, the reaction
products were electrophoresed on 15% SDS-PAGE and MBP
(at ~ 18 kDa) was visualized by autoradiography (B).

15 Cont/Control, cells grown overnight in serum-free
medium, and treated with 1% ethanol for 2 h.

Figure 5. Tempo stimulates tyrosine
phosphorylation and activity of SAPK. Cells were
grown in serum-free medium overnight, and treated with

20 tempol (10 mM) or tempo (10 mM) for indicated times,
followed by lysis. A. WCLs (1 mg) were
immunoprecipitated with agarose-conjugated anti-SAPK
antibody, followed by immunoblotting with anti-PY MAb.
B. In other independent experiments, anti-SAPK

25 immunoprecipitates were first probed with anti-PY MAb
(top), and the blot was then reprobed with anti-SAPK
antibody (bottom). C. Cells were grown in serum-free
medium overnight and treated with tempol (10 mM) or
tempo (10 mM) for 2 h. WCLs (1 mg) were

30 immunoprecipitated with anti-JNK1 antibody, and the
JNK1 activity in immunoprecipitates was measured using
GST-cJun (~ 41 kDa) as a substrate. UT, untreated
cells grown overnight in serum-free medium;
CONT/Control, cells grown in serum-free medium

35 overnight, and treated with 1% ethanol for 2 h.

8

Figure 6. Effects of tempol and tempo on cell
viability. Cells were grown in serum-free medium
overnight in 96-well plates, and treated with tempol
(10 mM) or tempo (10 mM) for indicated times, followed
5   by removal of medium containing the nitroxide
compound. Control cells were grown overnight in
serum-free medium, followed by treatment with 1 %
ethanol for various times. Fresh serum-free medium
(100 ul) was added to each well in all plates,
10 including controls followed by the addition of WST-1
(10 ul). Plates were incubated for 2 h at 37°C and
the color solution developed by WST-1 was quantified
using a MR 700 microplate reader at OD=450/600.
Values shown are mean ± s.d. of 6 determinations per
15 treatment condition in a representative experiment,
and the experiment was repeated three times.
Figure 7. Tempo induces apoptotic cell death.
Cells were grown in serum-free medium overnight in T-
25 flasks and treated with tempol (10 mM) or tempo (10
20 mM) for various times, trypsinized, and then
resuspended in 200 ul 1X binding buffer as described
in experimental procedures. The cell suspension was
double-stained with annexin V-FITC and propidium
iodide and analyzed by flow cytometry. Background
25 signal was determined by comparison with double-
stained, single-stained, or unstained control cells.
A, C, and E are cytograms showing a relative
distribution of viable (V), apoptotic (A), and
necrotic (N) cells at 2 h following tempol or tempo
30 treatment of MDA-MB 231 cells (A), PCI-04A cells (C),
and PC-3 cells (E). B, D, and F are time course
analyses of MDA-MB 231 cells (B), PCI-04A cells (D),
and PC-3 cells (F). 100,000 cells were analyzed at
each time point in triplicate (B) or quadruplicate (D
35 and F). Solid bars, 1% ethanol, striped bars, 10mM

tempol, empty bars, 10 mM tempo. A, The percentage of
MDA-MB 231 cells in each quadrant is: Control- V,
92.88%, A, 6.05%, N, 1.06%; Tempol- V, 92.29%, A,
6.32%, N, 1.31%; Tempo, V, 35.83%, A, 52.42%, N,

5   10.90%. Data shown are representative from three to
four independent experiments. B, Time course analysis
of MDA-MB 231 cells undergoing apoptosis (annexin V-
FITC staining), or necrosis (propidium iodide
staining). Values shown are mean ± s.d.of triplicate

10   determinations per time point in each treatment
category, and representative of three to four
independent experiments. C, The percentage of PCI-04A
cells in each quadrant is: Control, V, 87.64%, A,
3.42%, N, 8.45%; Tempol, V, 89.79%, A, 5.44%, N,

15   4.57%; Tempo, V, 18.61%, A, 48.35%, N, 31.62%. D,
Time course analysis of PCI-04A cells undergoing
apoptosis (annexin V-FITC staining), or necrosis
(propidium iodide staining). Values shown are mean ±
s.d.of quadruplicate determinations per time point in

20   each treatment category. E, The percentage of PC-3
cells in each quadrant is: Control, V, 96.61%, A,
1.12%, N, 1.95%; Tempol, V, 95.76%, A, 1.20%, N,
2.86%; Tempo, V, 10.76%, A, 2.57%, N, 83.52%. F,
Time course analysis of PC-3 cells undergoing

25   apoptosis (annexin V-FITC staining), or necrosis
(propidium iodide staining). Values shown are mean ±
s.d.of quadruplicate determinations per time point in
each treatment category. Control/C, cells treated
with 1% ethanol for 2 h.

30   Figure 8. Ceramide production in tempo-treated
MDA-MB 231 cells. Logarithmically growing cells were
cultured in serum-free medium overnight in 60 mm
dishes, and treated with tempol (10 mM) and tempo (10
mM) for indicated times, followed by lipid extraction,

35   and quantitation of ceramide by DAG kinase assay as

described in experimental procedures. The organic
phase extract containing the [$\gamma^{32}P$]-labeled ceramide
was quantitated. Control cells were grown overnight
in serum-free medium, followed by treatment with 1 %
5   ethanol for various times ranging from 0.5 h to 2 h.
Tempol/tempo treatment values shown are the mean ±
s.d. of triplicate determinations, the value at each
time point normalized to control (100%).

      Figure 9 shows the time-course and dose-response
10   experiments demonstrating tempo-induced apoptosis in
prostate cancer cells (A and B, DU145; C, PC-3; D,
LNCaP). Cells were grown overnight in medium
containing 10% BCS in T-25 flasks, switched to medium
containing 5% BCS and treated with tempo. Following
15   treatment, cells were washed, trypsinized, and
resuspended in 200 ml of 1X binding buffer as
described before. The cell suspension was double-
stained with annexin V-FITC and PI and analyzed by
flow cytometry. Background signal was determined by
20   comparison with double stained, single-stained, or
unstained control cells. A: Cytograms of untreated
(left panel); 0.1% ethanol-treated (24 hr) (middle
panel); and tempo-treated DU145 cells (2.5 mM, 24 hr)
(right panel). Each panel shows relative distribution
25   of viable (bottom left quadrant), apoptotic (bottom
right quadrant), and necrotic cells (top right
quadrant). B-D: apoptosis (right panels) and necrosis
(left panels) in cells treated with tempo (B, 2.5 mM;
C, 24 hr; D, doses and times as indicated). Values
30   shown are mean q S.D. of triplicate determination per
point in each treatment category.

      Figure 10 illustrates the effect of tempo on
caspase-3 activity in prostate cancer cell lines.
Cells were treated with 2.5 mM tempo for indicated
35   times in duplicate or triplicate as described in

legend to figure 2. Representative data from one
experiment are shown. Experiments was repeated 2-3
times.

Figure 11 shows phase contrast light microscopy
5   of LNCaP cells. LNCaP cells (1x106) were seeded in 25
cm2 tissue culture flasks in RPMI medium containing
10% BCS overnight, followed by change to RPMI medium
containing 5% BCS and stimulation with 0.1% ethanol
(control, left panels) or tempo (2.5 mM, right
10 panels) for indicated times.

Figure 12 displays transmission electron
microscopy of LNCaP cells. A1, untreated, 4,000x; A2,
a portion of the cell shown in A1, 20,000x; B1, 0.1%
ethanol, 24 hr, 4,000x; B2, a portion of the cell
15 shown in B1, 20,000x; C1, 5 mM tempo, 24 hr, 4,000x;
C2, a portion of the cell shown in C1, 8,000x; D, 5 mM
tempo, 24 hr, a portion of another cell, 20,000x. M,
mitochondria; G, golgi apparatus; RER, rough
endoplasmic reticulum; Cy, cytoplasm; N, nucleus;
20 Arrow in panel C2 indicates swelling in the nuclear
membrane.

Figure 13 illustrates the effects of tempo on the
growth of human breast tumor xenografts in athymic
mice. A. Tempo (200 mg/kg) was administered
25 intratumorally once daily for a total of 8 days (*).
Control group was treated simultaneously with an equal
volume of ethanol (10%). Experiment was terminated
when thecontrol tumor volumes exceeded the recommended
tumor burden. Each pointrepresents mean q S.E. (n=4).
30 Experiment was repeated twice with comparable results.
B. Representative tumor-bearing mice. Mice were
treated with 10%ethanol (left panel) or tempo (right
panel) as explained above.

Detailed Description

Tempo is a stable nitroxide free radical that is shown to have antioxidant catalytic activity which mimics those of superoxide dismutase (SOD), and which
5   when existing *in vivo*, can interact with other substances to perform catalase-mimic activity. In the past, nitroxides including tempo, have been used in electron spin resonance spectroscopy as "spin labels" for studying conformational and motional
10 characteristics of biomacromolecules. Nitroxides have also been used to detect reactive free radical intermediates because their chemical structure provides a stable unpaired electron with well defined hyperfine interactions. In addition, nitroxides have
15 been observed to act as enzyme mimics; certain low molecular weight nitroxides have been identified to mimic the activity of superoxide dismutase (Samuni, A et al., 1988, *J. Biol Chem.* 263, 17921) and catalase (Mehlhorn, R. J. et al., 1992, *Free Rad. Res. Comm.*
20 17, 157). Numerous studies also show that nitroxides that are permeable to cell membranes are capable of short-term protection of mammalian cells against cytotoxicity from superoxide anion generated by hypoxantine/xanthine oxidase and from hydrogen
25 peroxide exposure. The ability of tempo to induce cell death in vitro and in vivo is novel and unexpected. Delivery of tempo to the desired cells can be achieved by conjugating tempo to a marker specific to the desired cells. Such markers include
30 antibodies specific for such cells, growth factors for which the cells have receptors, or ligands which bind specifically to a factor on such cells. For example, HER/2neu ligands are selective for breast cancer cells.

By "tempo" is meant the stable nitroxide free radical, its precursor (such as the N--H form), and derivatives thereof including their corresponding hydroxylamine derivative (N--OH) where the oxygen
5 atoms are replaced with a hydroxyl group and exist in a hydrogen halide form, and the chloride salt form of the hydroxylamine derivative.

By "a functional derivative of tempo" is meant a natural or synthetic substituent, analog or derivative
10 of tempo which retains or contains the cytotoxic effect of tempo. Tempo could be delivered by suitable carriers such as liposomes. Tempo could also be conjugated with target tissue- or cell-specific markers such as antibodies or ligands. In addition,
15 tempo structure could be modified to improve upon its stability in target cells or tissues thereby allowing for a reduced administered concentration of tempo. The formation of substituent, derivatives or synthetic analogs is known in the art and the cytotoxic ability
20 of the compounds generated can be tested by methods known in the art including the assays described in the Examples below.

Tempo has a 6-membered heterocyclic structure in the form of 2,2,6,6-tetramethyl-1-piperidinyloxy, or
25 2,2,6,6-tetramethylpiperidin-N-oxyl. The substituent groups are usually methyl groups or ethyl groups, although other longer carbon chain species could be used. tempo can be substituted, typically in the 4 position, for example 4-amino, 4-(2-bromoacetamido),
30 4-(ethoxyfluorophosphonyloxy), 4-(2-iodoacetamido), 4-isothiocyanato, 4-maleimido, 4-(4-nitrobenzoyloxyl), 4-oxo, 4-phosphonooxy, and the like. Other natural or synthetic derivatives and precursors of tempo which result in a compound which effectively induces cell
35 death is part of this invention. Methods, such as

those described below and other methods, are known in the art for testing cytotoxicity of a compound. Persons with ordinary skill in the art recognize that by substituting various functional groups on the

5   nitroxide, it is possible to manipulate properties including solubility, biodistribution, *in vivo* stability, and tolerance.

In this invention is described a method for inducing cell death comprising administering to a cell

10   a composition comprising tempo or a functional derivative of tempo in an amount sufficient to induce death of said cell. The composition may further comprise an excipient or diluent or other medicament or treatment, or a molecule or carrier for the site

15   specific localization of tempo or its functional derivative. A carrier can be a biomolecule or a synthetic molecule such as dextran.

Results from experiments described below indicate that tempo induces cell death by activating the SAPK

20   or caspase-3 signaling cascade. Therefore, another embodiment of the present invention relates to a method for activating the SAPK signaling cascade or a caspase-3 cascade in a cell. The method comprises administering a composition comprising tempo to a cell

25   in an amount effective for activating the SAPK signaling cascade or caspase-3 signaling cascade in said cell.

A variety of techniques have been described to covalently attach a nitroxide to biomolecules,

30   including hemoglobin, albumin, immunoglobulins, and liposomes. See e.g. McConnell *et al.*, 1970, *Quart. Rev. Biophys.* 3, 91; Hamilton *et al.*, 1968, <u>Structural Chemistry and Molecular Biology</u>. A Rich *et al.*, eds. W. H. Freeman, San Francisco, p. 115; Griggith *et al.*,

1969, Acc. Chem. REs. 2, 17. Pursuant to this
invention, it is possible to select or design carriers
which can deliver tempo to particular sites in the
body as a means of localizing therapeutic, apoptotic
5 activity. Carriers include, but are not limited to,
liposomes since tempo is lipophilic. Targets include,
but are not limited to, tumor cells containing
specific ligands, receptor molecules, e.g. receptors
for growth factors, such as epidermal growth factor ,
10 Her-2/New, fibroblast growth factor; or cytokines such
as interleukins, interferons, and tumor necrosis
, factor.

        Diseases where selective cell death is beneficial
or can be part of the treatment of said disease
15 include diseases associated with abnomal cell
proliferation, such as warts, moles, and the like,
cancer, e.g. prostate, breast, ovary, head and neck,
kidney, lungs, bone, brain, pancreas, liver, or any
other disease where diseased cell death is beneficial.
20 The levels of tempo which may be administered
pusuant to this invention are well tolerated in
animals and are expected to be well tolerated in
humans. For example, a tolerated intraperitoneal dose
of tempo in mice is 5 mg/kg to 1000 mg/kg.
25 Intratumoral tolerance can be over 200 mg/kg.
Further, if the tempo is bound to a carrier and
injected intravenously, the carrier may serve to
confine the tempo to the vascular compartment, where
the utility is optimized, due to the membrane
30 impermeability of the carrier.

        When tempo is injected, it diffuses rapidly into
the intracellular space, where it is reduced to the
hydroxylamine form from an oxoammonium intermediate.
The hydroxylamine does not have the catalytic activity
35 of tempo. The hydroxylamine is chemically stable and

relatively persistant in the body and, in accord with
the teachings of this invention, can be chemically
converted back to the active from of the nitroxide.
This in vivo conversion enables the safe clinical use
5   of tempo to provide a sustained activity. When
conversion of hydroxylamine is selective, selective
cell death is possible.

In addition, this invention describes tempo-
containing compounds that are associated with a
10   container for storage or administration of
pharmaceuticals such as intravenous fluids, topical
agents and others. In view of the stable chemical
nature of tempo, compositions containing tempo can be
administered by various routes. Tempo can be
15   administered parentarally or orally. In the reduced
form, hydroxylamine, can act locally in the
gastrointestinal system or be taken up into the blood.
Thus, sustained activity can be provided in all body
compartments. Tempo complexed to a macromolecule can
20   be administered parenterally where it will remain
localized in the extracellular space thereby providing
a localized effect.

With respect to selecting a particular
formulation and method of administration pursuant to
25   this invention, the formulation and method of
administration are dictated by the particular
application. The selection of a tempo-based compound
is based on the site where activity is desired. Where
specific activity is desired in the gastrointestinal
30   tract, a tempo-dextran complex is preferred because
such a compound is less susceptible to enzymatic
digestion while in the gastrointestinal tract. In
such an application, oral or rectal administration is
preferred. Where specific activity is sought for the
35   intravenous or intravascular regions, such as the

cardiovascular system, a tempo-albumin complex is
preferred because albumin is a major plasma protein,
is well-tolerated, easy to administer, and exhibits an
extended plasma half-life. The same rationale applies
5   for intraperitoneal or intradermal administration. If
specific activity in the lungs is desired, an aerosol
from of tempo of tempo-albumin complex is preferred.
As will be apparent to those skilled in the art, these
preferred formulations may be altered depending on the
10   particular application. Tempo can be administered
intratumourally. The dosage will depend upon the
disease indication and the route of administration but
should be between 5-2000 mg/kg of body weight/day.
The duration of treatment will extend through the
15   course of the disease symptoms, possibly continuously
using a slow-releasing pump or formulation, or single
or multiple bolus intratumoral injections. Multiple
doses may be required, the number of doses depending
upon disease delivery vehicle and efficacy data from
20   clinical trials.

        The formulation or method of administration
should achieve a systemic or tissue specific
distribution commensurate in scope with the extent of
the disease or the region to be treated.
25   The formulations of the invention may also
include additional solvents and/or carrier materials
and/or extenders such as alcohols, e.g. ethanol,
water, sodium chloride or dextrose or other
pharmaceutically acceptable solvents used for
30   systemic, including oral or parenteral,
administration.

        In carrying out the method of the present
invention, tempo in combination with the solvent or
carrier or optionally an additional pharmaceutical
35   therein, or tempo alone, may be administered to

mammalian species, such as monkeys, dogs, cats, rats,
humans, etc.. Methods of adminstration include but
are not limited to oral, intradermal, transdermal,
intravenous, subcutaneous, intramuscular,

5   intraperitoneal, and intranasal routes. Such
administration can be done in either bolus or repeat
doses or continuously by infusion for instance.

Where tempo alone or in combination with any of
the other components of the formulation of the

10   invention is to be administered by angiography or
intracoronary injection, it (or the combination) will
be formulated in a conventional vehicle, such as
distilled water, saline, Ringer's solution, or other
conventional carriers.

15   Tempo alone or in combination with any of the
other components of the formulation of the invention
may also be incorporated in a conventional dosage
form, such as a tablet, capsule or elixir or
injectable. The above dosage forms will also include

20   the necessary carrier material, excipient, lubricant,
buffer, antibacterial, bulking agent (such as
mannitol), anti-oxidants (ascorbic acid of sodium
bisulfite) or the like. Parenteral dosage forms are
preferred, although oral forms may be satisfactory as

25   well.

A pharmaceutical kit comprising one or more
containers filled with one or more of the tempo
compositions can be included along with containers
containing the solvent or carrier and other necessary

30   reagent or reagents for mixing or dissolving any of
the components of the kit.

All documents cited herein *supra* and *infra* are
hereby incorporated in their entirety by reference
thereto.

This invention can be better understood by referring to the following examples which are given for illustrative purposes only and are not meant to limit the invention.

5   The following MATERIALS AND METHODS were used in the examples that follow.

Antibodies and Reagents - The following antibodies were used in thisstudy: anti-SAPK polyclonal antibody (α-NT),   anti-phosphotyrosine

10   monoclonal(MAb) antibody (α-PY, 4G10), and agarose conjugated α-PY (UBI, Lake Placid, NY); agarose conjugated anti-ERK-1 (C-16, sc-93ac), anti-JNK1 (C-17, sc-474ac), and anti-Raf-1 (C-12) polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa

15   Cruz, CA); anti-Raf-1 MAb (c-Raf-1) and anti-ERK-1 MAb (MK12) (Transduction Laboratories, Lexington, Kentucky). Protein A-agarose and Syntide-2 were obtained from Santa Cruz Biotechnology,Inc. (Santa Cruz, CA). The nitroxide compounds tempo (2,2,6,6-

20   tetramethylpiperidine-N-oxyl) and tempol (4-hydroxy-tempo) were obtained from Aldrich Chemical Co (Milwaukee, WI). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents and 5X TGS electrophoretic buffer were purchased from

25   Gibco BRL (Grand Island, NY), and pre-mixed 10X Tris-glycine transfer buffer was obtained BioRad Laboratories (Hercules, CA). All other reagents were obtained from Sigma (St. Louis, Missouri) unless otherwise indicated.

30   Cell Culture, Treatments with Tempol and Tempo, and Preparation of Cell Lysates - MDA-MB 231 human breast cancer cells were grown to near confluence in 75 cm^2 tissue culture flasks in Improved Minimum Essential Medium (IMEM) (Cellgro) containing 10% fetal

35   bovine serum (FBS) and 2 mM L-glutamine in a

humidified atmosphere of 5% CO_2: 95% air at 37°C.
Cells were trypsinized and plated onto a 150 mm tissue
culture dish (two dishes per flask) overnight in
medium containing 10% FBS followed by two washes with
5 phosphate buffer saline (PBS). Cultures were
maintained in serum-free medium overnight prior to
tempol (10 mM) or tempo (10 mM) treatment. Both
nitroxide radicals were dissolved in ethanol before
use. For the extraction of whole cell lysates (WCL),
10 cells with or without nitroxide treatment were washed
three times with ice-cold PBS containing 0.5 uM sodium
orthovanadate (Na_3VO_4) and lysed in lysis buffer (50
mM HEPES, pH 7.5, 1% Nonidet P-40 (NP-40), 10%
glycerol, 4 ug/ml each of leupeptin, aprotinin and
15 pepstatin A, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl
fluoride (PMSF), 25 mM sodium fluoride (NaF), and 0.5
mM EDTA. WCL was agitated for 1 h at 4°C and
centrifuged in a microcentrifuge at 15,000 x g, 4°C
for 15 min to remove cellular debris. The supernatant
20 was aliquoted and stored at -70°C until use.
     Immunoprecipitation and Immunoblotting –
Whole cell lysate (1 mg) was immunoprecipitated with
the appropriate agarose-conjugated antibody (1 ug/ml
of lysis buffer) overnight at 4°C with constant
25 agitation. For SAPK immunoprecipitation, WCL (1 mg)
was immunoprecipitated with anti-SAPK antibody (5
ug/ml) overnight followed by addition of protein A-
Agarose (50 ul of 250 ul/ml stock) and incubation for
2 h at 4°C. Immune-complex beads were collected by
30 microcentrifugation at 15,000 x g for 5 min followed
by three washes with lysis buffer. The beads were
resuspended in 2X electrophoresis sample buffer,
boiled for 5 min, and proteins were resolved by 10%
SDS-PAGE and transferred to an Immobilon-P membrane.
35 The membrane was blocked with 4% bovine serum albumin

(BSA) in PBS-Tween (0.25%), and immunoblotted with the
desired primary antibody at 1:2000, followed by
1:10,000 dilution of an appropriate horseradish
peroxidase-coupled secondary antibody. The
5   immunoreactive protein bands were revealed by ECL
detection system (Amersham, Arlington Heights, IL).
The bands of interest were quantified by ImageQuant
software version 3.3 (Molecular Dynamics Personal
Densitometer, Sunnyvale, CA). Prior to reprobing,
10   blots were stripped according to the ECL kit protocol
(NEN, Boston, MA), as described earlier (Suy, S. et
al., 1997, *Oncogene* 15, 53-61).
       Raf-1 kinase Assay - Raf-1 protein kinase
activity was measured by a kinase cascade assay
15   according to the manufacturer's procedure (UBI, Lake
Placid, NY), with the following modifications.
Briefly, Raf-1 immune-complex was washed 3 times with
lysis buffer and once with kinase binding buffer (KBB)
(20 mM MOPS, pH 7.2, 25 mM-glycerol phosphate, 5 mM
20   EGTA, 1 mM Na_3VO_4, 1 mM DTT). This was followed by
incubation of the immune-complex for 30 min at 30°C
in reaction mixture containing 20 ul of KBB, 10 ul of
0.5 mM ATP/Mg cocktail (75 mM magnesium chloride and
500 uM ATP in KBB), 1.6 ul of inactive MAPK Kinase
25   (0.4 ug) and 4 ul of inactive MAPK (1 ug). At the end
of the reaction, 8 ul of the sample mixture was
transferred to fresh 1.5 ml microfuge tube, followed
by the sequential addition of 10 ul KBB, 10 ul of MBP
substrate (2 mg/ml stock), and 10 ul of [γ-^{32}P] ATP (1
30   uCi/ul generated by 1:10 dilution of the stock 3000
Ci/mmole (Dupont NEN, Boston, MA) in ATP/Mg^{2+}
cocktail). This reaction mixture was incubated for 10
min at 30°C. The immune-complex was then pelleted by
brief centrifugation in a bench-top microcentrifuge,
35   and 5 ul of the sample was spotted, in triplicate,

onto P81 paper. The radioactive filters were
transferred onto a 50 ml conical tube (20 filters per
tube) and washed 4 times with 40 ml of 0.75%
phosphoric acid (15 min each), followed by a brief
5   acetone wash, and counted using Beckman LS 1801
scintillation counter.

Additionally, the immune-complex-associated Raf-1
activity was measuredusing Syntide-2 as a substrate.
This reaction was initiated by sequentialaddition of
10   15 ul KBB, 10 ul of ATP/Mg^{2+} cocktail, 5 ul of Syntide-
2 (5 ug), and 10 ul of diluted [γ-^{32}P] ATP followed by
incubation of the reaction at 30°C for 20 min. At the
end of the incubation, reaction mixture was
centrifugedbriefly in a bench-top microcentrifuge, and
15   5 ul of the supernatant was spotted in triplicate onto
P81 filter paper, air dried, washed, and counted as
described above.

ERK and SAPK/JNK Activities - Whole cell lysates
prepared as described above were immunoprecipitated (1
20   mg) with an agarose conjugated anti-ERK1 antibody or
an agarose conjugated anti-JNK1 antibody for 2 h at
4°C with constant agitation. The immune-complexes
were washed 3 times in lysis buffer and once in KBB as
mentioned earlier. ERK or JNK activity assay was
25   carried out according to manufacturer's procedures
(UBI). Briefly, the ERK1 immunoprecipitates were
incubated for 10 min at 30°C in a kinase reaction
containing 10 ul of MBP (myelin basic protein) as
substrate (2 mg/ml stock), 10 ul of inhibitor cocktail
30   (20 uM PKC inhibitor peptide, 2 uM protein A inhibitor
peptide, and 20 uM compound R24571), and 10 ul of
magnesium-ATP cocktail (1·uci [γ-^{32}P] ATP generated by
1:10 dilution of stock (3000 Ci/mmol) in 75 mM
magnesium chloride and 500 um cold ATP). The immune-
35   complexes were centrifuged briefly in a bench top

centrifuge and 5 ul aliquots of the supernant were
spotted in triplicate onto P81 filter papers. The
radioactive filters were washed and counted as
described above. Alternatively, to visualize the
5   incorporation of [γ-^{32}P] into MBP, the kinase reaction
was stopped by addition of 2X electrophoresis sample
buffer, boiled for 5 min and proteins were resolved by
15% SDS-PAGE, followed by autoradiography. For JNK
activity assay, JNK1 immunoprecipitates were
10   incubated for 30 min at 30°C in 40 ul of kinase
reaction mixture containing 10 ul of KBB, 20 ul of the
GST-cJun fusion protein (0.2 ug/ul stock), and 10 ul
of the diluted [γ-^{32}P]ATP as described above. The
kinase reaction was stopped with 2X electrophoresis
15   sample buffer, boiled for 5 min, and the supernatant
was electrophoresed on a 12.5% SDS- PAGE. The
radiolabeled GST-cJun fusion protein was detected by
autoradiography.
     Cell Viability Assay - Effects of nitroxide
20   compounds on cell viability and proliferation were
determined using a cell viability detection kit (4-[3-
(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio] -
1,3-benzene disulfonate, WST-1) according to the
manufacturer's instructions (Boehringer Mannheim,
25   Indianapolis, IN). Briefly, MDA-MB 231 cells were
seeded onto 96 well plates at density of 10,000 cells
per well and maintained overnight in 10% FBS
containing medium. The cells were then washed twice
with phosphate buffered saline and refed in serum-free
30   medium. The following day, cells were treated for
various times with tempol (10 mM) or tempo (10 mM),
using six wells per treatment condition. At the end of
treatment, medium containing the nitroxide compound
was removed and replaced with fresh serum-free medium
35   (100 ul), followed by addition of WST-1 (10 ul).

Plates were incubated for 2 h at 37°C and analyzed at
OD=450/600 using a MR 700 microplate reader.

Apoptosis Assay - ApoAlert Annexin V apoptosis
detection system (Clontech Lab Inc., Palo Alto, CA)

5   was used to measure the relative distribution of
apoptotic and necrotic cells. Briefly, cells were
seeded at a density of 1 x 106 cells per 25 cm^2 tissue
culture flask in medium containing 10% FBS overnight,
followed by washing twice in serum-free medium. Cells

10 were maintained overnight in serum-free medium and
exposed to tempol (10 mM) or tempo (10 mM) for various
times. This was followed by rinsing twice with serum-
free medium prior to trypsinization, dilution in two
volumes of serum-free medium, and centrifugation at

15 10,000 x g. The cell pellet was washed once with PBS
and resuspended in 200 ul of 1X binding buffer. The
cell suspension was double-labeled with fluorescein
isothiocyanate (FITC)-labeled annexin V (10 ul) and
propidium iodide (PI) (10 ul) according to the

20 manufacturer's instructions. Unlabeled cells or
untreated cells labeled with either FITC-annexin V or
PI, or double-labeled served as internal controls for
the background signal. The intensity of the dye
uptake by cells was detected using FACStar plus Flow

25 Cytometer (Becton Dickerson, Lincoln Park, NJ), and
data were analyzed using Reproman True Facts software
(Seattle, Washington). Viable cells were FITC-/PI-,
apoptotic cells were FITC+/PI-, and necrotic cells
were FITC+/PI+.

30   Ceramide Generation Assay - Ceramide production
in MDA-MB 231 cells was determined by diacylglycerol
(DAG) kinase assay according to a previously
described procedure (Haimovitz-Friedman, A. et al.,
1994, J. Exp. Med. 180, 525-535; Dressler, K. A. and

35 Kolesnick, R. N., 1990, J. Biol. Chem. 265, 14917-

14921). Briefly, MDA-MB 231 cells were split (1:2),
and after 24 h, the cells were washed twice with PBS
and serum-free medium was added, followed by
incubation for additional 24 h. The cells (~2x10^6/60
5   mm dish) were treated with tempo (10 mM) or tempol (10
mM) for various times. Following treatment, floating
cells were collected and pelleted by centrifugation
for 10 min at 1200 rpm, and attached cells were
collected by scraping. Lipids were extracted from all
10   cells (floating and attached) by incubation in 1 ml
100% ice-cold methanol. After a partial purification
with chloroform, the extracted lipid in the organic
phase was dried under N$_2$ and was treated with a mild
alkaline solution (0.1 N KOH in methanol) for 1 h at
15   37°C to remove glycerolphospholipids. The organic
phase extract was resuspended in 20 ul of 7.5% n-
octyl-β-D-glucopyranoside, 5 mM cardiolipin, 1 mM EDTA
followed by the addition of 40 ul of purified DAG
kinase in DAG kinase buffer (20 mM Tris-HCl (pH 7.4),
20   10 mM DTT, 1.5 M NaCl, 250 mM sucrose, 15% glycerol).
The kinase reaction was initiated by the addition of
20 ul of diluted [γ-^{32}P]ATP (10 mM at 1,000 dpm/pmol in
DAG kinase buffer) and incubated for 30 min at 22°C.
This reaction was terminated by extraction of lipids
25   with 1 ml CHCl$_3$:CH$_3$OH:HCl (100:100:1), 170 ul buffered
saline solution (135 mM NaCl, 1.5 mM CaCl$_2$, 0.5 mM
MgCl$_2$, 5.6 mM glucose, and 10 mM HEPES, pH 7.2), and
30 ul of 100 mM EDTA. The lower organic phase
containing ceramide-1-phosphate was collected and
30   dried under N$_2$ followed by spotting and run of 40 ul
(80%) onto a thin layer chromatographic (TLC) (Whatman
silica gel 150A) plate, and developing in chamber
containing CHCl$_3$:CH$_3$OH:HAc (65:15:5, vol/vol) as
solvent. The spot containing the ceramide-1-phosphate
35   was visualized by autoradiography, and the

incorporated ^{32}P was removed by scraping, and
quantified by Cerenkov counting. A standard curve
consisting of known amount of ceramide was used as a
comparison to the level of observed ceramide
5   generated in MDA-MB 231 cells.

Caspase-3 activity assay:

ApoAlert CPP32 activity/caspase-3 assay kit was
used to measure caspase-3 activity in cells according
to manufacturer's instructions (Clontech). Briefly,
10   after tempo treatment, cells were washed 3 times with
ice-cold PBS and lysed in Clontech cell lysis buffer
for 10 min on ice followed by microcentrifugation at
15,000xg for 5 min. Whole cell lysate (50 ug) was
incubated for 1 h at 37°C in caspase-3 reaction
15   mixture containing 10 mM DTT in 2X reaction buffer, 50
uM CPP32 substrate, DEVD-AFC. The amounts of 7-amino-
4 trifluoromethyl coumarin (AFC) released were
detected by spectrofluorometer (Hitachi F4500) with
excitation at 400 nm and emission at 505 nm.
20   Transmission electron microscopy:

LNCaP cells were treated with tempo as described
in legend to figure 2. Following treatment, monolayer
cells were washed three times with PBS, and fixed in
2.5% glutaraldehyde/3% paraformaldehyde in PBS. After
25   fixation, the cell monolayer was washed in PBS. The
cells were collected by gentle scraping using a rubber
policeman, and centrifuged. The pellet was embedded
in 1% agarose to facilitate handling. Post-fixation
was performed in 1% osmium tetroxide in distilled
30   water for 1 hr, followed by washing three times in
distilled water, enbloc staining with 2% uranyl
acetate for 30 min in dark, and washing three times in
distilled water. This was followed by processing for
conventional ultrathin section electron microscopy.
35   Sections were mounted on 200 mesh-nickle grids, post-

stained with lead citrate, and photographed with a
JEOL 1200EX-transmission electron microscope operated
at 60kV.

Tumor growth studies:

5   MDA-MB231 cells (1x10^6) were inoculated
subcutaneously in the right flank of 4-6 week-old
female athymic mice. Tumor growth was monitored
biweekly. Mice bearing tumor volumes in the range of
80 - 100 mm^3 were randomly selected for treatment.

10   Tempo treatment was initiated (50 mg/kg-200 mg/kg,
intratumoral, once daily, for a total of 8 days), and
tumor volumes were measured for a total 25 days from
the start of treatment.

Example 1

15   Effects of Tempol and Tempo on Protein Tyrosine
Phosphorylation

Figure 2 illustrates a conspicuous increase in
the tyrosine phosphorylation of several as-yet
unidentified protein bands within 15 min after the

20   exposure of MDA-MB 231 cells to 10 mM tempo. These
levels remained elevated for the duration of the study
(2 h). In parallel experiments, minimal protein
tyrosine phosphorylation was observed at various times
(15 min to 2 h) following the treatment of cells with

25   an equimolar concentration of tempol. These data show
that while both nitroxides induced protein tyrosine
phosphorylation, the magnitude of this response was
clearly higher in tempo-treated cells.

Example 2

30   Tempol and Tempo Stimulate Tyrosine
Phosphorylation and Activity of Raf-1 In Vivo

Previously we demonstrated that ionizing
radiation, a well known stress inducing agent, causes
tyrosine phosphorylation of Raf-1 in MDA-MB 231 breast

cancer cells (Suy, S. et al., 1997, supra). Here we
examined the possibility of tyrosine phosphorylation
and activation of Raf-1 protein kinase in response to
tempol or tempo. Interestingly, both tempol and tempo

5   treatments led to an increase in the level of tyrosine
phosphorylated Raf-1 (Raf^P) (Figure 3A, top panel).
The level of total Raf-1 protein remained unchanged
(Figure 3A, bottom panel). The immunoreactive RafP
bands were quantified. Densitometric analysis

10   indicated that increase in the level of RafP detected
at 15 min was ~5- 8 fold, and Raf^P content was
comparable to the basal level by ~ 60 min to 120 min
(data not shown). The activity of Raf-1 protein kinase
was determined by a kinase cascade assay or by the

15   Syntide-2 phosphorylation assay (Figure 3B). In
agreement with the enhanced tyrosine phosphorylation
of Raf-1, tempol or tempo treatment resulted in ~ 2-3
fold increase in the Raf-1 protein kinase activity.

Example 3

20   <u>Tempol Stimulates ERK Activity</u>

Since Raf-1 activation, generally, leads to ERK
(p42/44 MAPK) activation, we examined the effects of
tempol and tempo on ERK1 enzymatic activity.
Representative experiments are shown in Figure 4

25   (panels A and B). Approximately 3-fold increase in
the enzymatic activity of ERK1 was detected by 2 h in
cells treated with tempol (Figure 4A). Interestingly,
however, no change in ERK1 activity was noted
following tempo treatment compared with control cells

30   (Figure 4A). In addition, ERK1 phosphorylation was
seen as a shift to a more slowly migrating
phosphorylated form (ERK1^P) on immunoblots using ERK1
immunoprecipitates at 2 h after tempol exposure (10
mM), but we were unable to identify a shift in the

mobility of ERK1 in tempo-treated cells (data not
shown).

Example 4

Tempo Treatment Results in Enhanced

5   Phosphorylation and Activation of SAPK In Vivo

We next measured the effects of tempol and tempo
on SAPK/JNK, a well-known component of the stress-
induced signal transduction pathway. The time course
experiments indicated that tempo treatment resulted in

10 a significant increase in the level of phosphorylated
SAPK (~ 54 kDa, SAPK^P) compared to tempol treatment
or untreated controls (Figures 5A and 5B). Consistent
with these data, SAPK enzymatic activity was
significantly induced in tempo-treated cells as shown

15 by the level of phosphorylated GST-cJun (Figure 5C).
Densitometric analysis of three independently
performed experiments indicated a 3 -7 fold increase
in the phosphorylated GST-cJun fusion protein
detectable after tempo exposure (10 mM, 2 h) compared

20 to tempol (10 mM, 2 h) or control (1% ethanol, 2 h)
treatment.

Example 5

Tempo Induces Apoptotic Cell Death

Several studies have reported that activation of

25 the SAPK signaling cascade is associated with
induction of apoptotic cell death (Kyriakis, J. M. and
Avruch, J., 1996, *supra*). To examine the possible
cytotoxic effects of tempo, we first used a
colorimetric assay to determine the cell viability and

30 proliferation. Treatment of cells with 10 mM tempo
resulted in > 50% decrease in the number of viable
cells within 2 h. In parallel experiments, the number
of viable cells in cultures treated with 10 mM tempol
was comparable to control cells treated with 1%

ethanol (Figure 6). These observations prompted us to
evaluate whether decrease in the number of viable
cells following tempo treatment was due to apoptotic
and/or necrotic cell death.

5   Apoptosis is a process of cell death
characterized by cytoplasmic shrinkage, nuclear
condensation, and DNA fragmentation (Kerr, J. F. R.,
et al., 1972, Br. J. Cancer 26, 239-257). Several
reports suggest that an early event leading to

10   apoptosis is accompanied by a loss of cell membrane
phospholipid asymmetry as a result of translocation of
phosphatidylserine (PS) from the intracellular
membrane to the extracellular membrane while leaving
the cell membrane intact (Fadok, V. A. et al., 1992,

15   J. Immunol. 148, 2207-2216). A PS-binding protein,
annexin V, has been used as a specific probe to detect
externalization of this phospholipid in a variety of
murine and human cell types undergoing apoptosis
(Martin, S. J. et al., 1995, J. Exp. Med. 182, 1545-

20   1556; Koopman, G. et al., 1994, Blood 84, 1415-1420).
Cell necrosis, on the other hand, is associated with
both the translocation of PS to the external cell
surface as well as the loss of membrane integrity
(Vermes, I. et al., 1995, J. Immunol. Methods 184, 39-

25   51). The cell membrane integrity of apoptotic cells
can be established with a dye exclusion test using
propidium iodide (PI). In the following experiments,
we used FITC-conjugated annexin V and propidium iodide
(PI) as markers for the evaluation of apoptosis and

30   necrosis. MDA-MB 231 cells treated with tempol or
tempo were double-labeled with FITC-conjugated
annexin V and propidium iodine (PI) and then subjected
to flow cytometric analysis. Representative cytogram
analysis of MDA-MB 231 cells with or without nitroxide

35   compound is shown in Figure 7A. The lower left

quadrant represents viable cells (V) which were
negative for annexin V and PI. The lower right
quadrant represents apoptotic cells (A) which were
positive for annexin V staining. The upper right
5   quadrant represents necrotic cells (N) which were
positive for both annexin V and PI stains. Tempo
treatment (10 mM, 2 h) resulted in a significant
increase in both the annexin V uptake (52.42%
apoptotic cells) and the annexin V plus PI uptake
10   (10.90% necrotic cells) compared to tempol (10 mM, 2h)
(6.32% apoptotic cells, 1.31% necrotic cells) and
control cells (1% ethanol, 2h) (6.05% apoptotic cells,
1.06% necrotic cells). Time course analysis indicated
that tempo treatment resulted in a steady increase in
15   the number of apoptotic cells for upto 2 h, followed
by a considerable increase in the number of necrotic
cells by 3 h (Figure 7B). Tempol treatment did not
induce apoptosis or necrosis for the duration of the
study (3 h) (Figure 7B). These data suggest that
20   tempo-stimulated SAPK phosphorylation and activation
may be associated with apoptotic cell death in MDA-MB
231 cells.

To determine the generality of the cytotoxic
effect of tempo in cancer cells, we have examined two
25   other cancer cell lines: PCI-04A, a human laryngeal
squamous carcinoma-derived cell line (Heo, D. S. et
al., 1989, Cancer Res. 49, 5167-5175), and PC-3, a
human prostate cancer cell line. The data shown in
Figures 7C and 7D demonstrate a significant level of
30   apoptosis and necrosis at 2 h post-tempo treatment (10
mM) in PCI-04A cells. In PC-3 cells, 10 mM tempo
treatment resulted in ~ 84% necrotic cells by 2 h,
implying that this treatment condition was highly
toxic (Figures 7E and 7F). Tempo also induced
35   apoptotic cell death in bovine aortic endothelial

cells, as measured by the bisbenzamide
trihydrochloride/Hoechst-33258 staining method, as
previously described (Haimovitz-Friedman, A. et al.,
1994, *J. Exp. Med.* 180, 525-535) (control: 4h, 1.55 ±
5   0.02%; 8 h, 1.99 ± 0.43%; tempo (5 mM): 4 h, 3.97 ±
0.33%; 8 h, 38.85 ± 1.69%) (Suy, S. et al., 1998, J.
Biol. Chem. . These results clearly demonstrate that
tempo but not tempol induces cell death in different
types of cancer cells.

10   **Example 6**

Ceramide Generation in Tempo-treated MDA-MB 231
Cells - Ceramide, a second messenger molecule
generated as a result of hydrolysis of the plasma
membrane phospholipid sphingomyelin or via *de novo*
15   synthesis, has been implicated in a variety of
biological responses to environmental cues (Kolesnick,
R. N., 1992, *Trends Cell Biol.* 2, 232). Increase in
ceramide has been correlated with increased JNK/SAPK
activity, and ceramide and SAPK/JNK have been shown to
20   participate in a signal transduction pathway leading
to cell death (Verheij, C. et al., 1996, *supra*;
Westwick, J. K. et al., 1995, *supra*; Yan, M. et al.,
1994, *Nature* 372, 798-800; Zanke, B. W. et al., 1996,
Curr. Biol. 6, 606-613). To assess the possibility of
25   a role of ceramide in tempo-induced SAPK and
apoptosis, we used a DAG kinase assay to quantify the
ceramide levels in MDA-MD 231 cells treated with or
without the nitroxide compound. A 54% increase over
control (normalized to 100%) in ceramide level was
30   observed at 30 min, and ceramide level reached 71%
over control at 1 h post-tempo treatment (Figure 8).
The level of ceramide generated in tempol-treated
cells was not significantly higher compared to control
cells at all time points. Ceramide production

preceeded maximal stimulation of JNK/SAPK and
apoptosis, implying its involvement in tempo-induced
signaling in MDA-MB 231 cells.

Example 7

5   To invesitgate tempo's effect on prostate cancer
cells, human androgen-independent prostate cancer-
derived cell lines (DU145 and PC3) were grown to near
confluence in 75cm^2-tissue flasks in Improved Minimum
Essential Medium (IMEM) (Cellgro) containing 10%
10   bovine calf serum (BCS) supplemented with 2 mM L-
glutamine and 200 IU/ml penicillin and streptomycin
mixture in a humidified atmosphere of 5% CO_2:95% air
at 37°C. Human androgen-dependent prostate cancer-
derived cell line (LNCaP) was cultured in RPMI 1640
15   medium containing 10% BCS and 2 mM L-glutamine. Cells
were trypsinized and seeded in equal numbers overnight
in 150 mm^2 tissue culture dishes (two dishes per flask)
or 25 cm^2 flasks (1x10^6 cells per flask). Cells were
treated in medium containing 5% BCS with desired
20   concentration of tempo for various times. Tempo was
dissolved in ethanol (0.1%) before use. Control
cultures were treated with ethanol (0.1%) for various
times. Following treatment, cells were washed 3 times
with PBS. ApoAlert Annexin V apoptosis detection
25   system (Clontech) was used to measure the relative
distribution of apoptotic and necrotic cells in
response to tempo as described earlier. Results of
flow cytometric analysis of cells labeled with FITC-
conjugated annexin V or propidium iodide showed that
30   tempo treatment led to significant levels of apoptosis
in these prostate tumor cell lines. In DU145 cells
and PC-3 cells, 2.5 mM tempo treatment for 24 hr
resulted in approximately 3.4-fold and 6.7-fold
increases in the number of apoptotic cells,
35   respectively. In LNCaP cells, a relatively higher

level of apoptosis was observed (1 mM tempo, 24 hr, approximately 12-fold; 5 mM tempo, 4 hr, approximately 15-fold) (Figure 9).

Example 8

5   Activation of the caspase family of cysteine proteases is an important molecular hallmark of programmed cell death. Caspase-3 is a well known downstream effector of apoptotic signal transduction pathway induced by a variety of agents. We asked
10   whether tempo activates caspase-3 activity in prostate cancer cells. As shown in Figure 10, tempo induced the activation of caspase-3, albeit to varying levels in different prostate cancer cell lines. In DU145 and PC3 cells, only modest increases in the level of
15   caspase-3 activity were observed with tempo (2.5 mM) (DU145, 2 hr, ~ 170%; PC3, 24 hr, ~ 200%). Consistent with a significant tempo-induced apoptosis in LNCaP cells, ~ 12-fold increase in caspase activity was noted in tempo-treated LNCaP cells (2.5 mM, 24 hr).

20   ### Example 9

       At the light microscopic level, a distinct change in cell morphology from spindle shaped, highly refractile appearance to a more flattened appearance was noticed after tempo treatment of LNCaP cells
25   (Figure 11).

                           ### Example 10

       We then undertook the ultrastructural analysis to examine if tempo induces the morphological features of apoptosis. Electron microscopy revealed aggregation
30   and marginalization of chromatin in the nuclei of a large number of tempo-treated LNCaP cells. The nuclear envelope remained essentially intact. In the cytoplasm, the golgi apparatus and rough endoplasmic reticulum had disappeared or disrupted, and
35   mitochondria were not discernible in a majority of

tempo-treated cells. Interestingly, mitochondria in
0.1% ethanol-treated cells were swollen compared to
the untreated controls. Pronounced vacuolation,
perhaps due to dilated endoplasmic reticulum or golgi

5   vesicles, was seen throughout the cytoplasm in more
than 90% of tempo-treated cells, making it very
difficult to identify membranous organelles. Recent
evidence indicates that membrane blebbing and caspase
activation are not in the same linear cascade of

10   apoptosis (Huot, J. et al., 1998, J. Cell Biol. 143,
1361-1373). Consistent with this information, bleb
formation was not noticed in tempo-treated cells.
Together, these findings support the view of tempo-
induced apoptosis in LNCaP cells.

15   Example 11
      A significant decline in tumor growth rate was
noted in tempo-treated group as compared to control
vehicle group (10% ethanol). These in vivo data
suggest the possibility of an anti-tumor activity of

20   tempo (Figure 13).

                           DISCUSSION
      This study reports, for the first time to our
knowledge, signal transduction mechanisms of cellular
response to two nitroxides, tempol and tempo, well

25   known for their antioxidant properties. Initially, we
hypothesized that since ERK pathway is used by a wide
variety of cell types for transducing survival or
proliferative signals, the antioxidant effects of
tempol and tempo may be complemented by stimulation of

30   the ERK-signaling pathway. Previous in vitro studies
suggested that at least 5-10 mM tempol is required to
provide radioprotection, and protection factor as high
as 2.2 was achieved with 100 mM tempol (15). Our data
showing activation of ERK1 by tempol (10 mM) is

35   consistent with these and other reports of a

protective role of tempol against radiation-induced
mutagenicity and double strand breaks, and hydrogen
peroxide induced mutagenicity (Hahn, S.M., 1992,
supra; Hahn, S. M. et al., 1994, *Cancer Res.* 54,
5   (suppl.) 2006s-2010s; DeGraff, W. G. et al., 1992,
Environ. Mol. Mutagen. 19, 21-26; DeGraff, W. G. et
al., 1992, *Free Radical Biol. Med.* 13, 479-487).
Surprisingly, however, tempo (10 mM) had no detectable
effect on ERK1 activity, suggesting that a
10   dissociation may also exist between ERK-signaling and
antioxidant activity of certain nitroxides.

Enhanced protein tyrosine phosphorylation,
generation of ceramide, activation of SAPK and
induction of apoptosis by tempo are unexpected and
15   novel observations. One possibility for further
evaluation is that there may be differential
intracellular reduction rate of tempol vs. tempo. In
this situation, tempo-treated cells may have higher
tempo free radical concentration. Free radicals, as
20   second messengers, would then find appropriate
cellular targets and turn on a signaling pathway. In
this context, it is noteworthy that addition of
platelet-derived growth factor (PDGF) to vascular
smooth muscle cells results in increased intracellular
25   levels of hydrogen peroxide and reactive oxygen
species (ROS), and these events have been correlated
with PDGF-induced tyrosine phosphorylation, MAPK
stimulation, and DNA synthesis (Sundaresan, M. et al.,
1995, *Science* 270, 26-299). In other reports,
30   induction of protein tyrosine phosphorylation in
neutrophils is dependent on NADPH oxidase activation
(Fialkow, L. et al., 1993, *J. Biol. Chem.* 268, 17131-
17137), and stimulation of as-yet unidentified protein
tyrosine kinases has been linked to apoptotic death of

B-lymphocytes (Uckun, F. M. *et al.*, 1992, *Proc. Natl.*
Acad. Sci. U.S.A. 89, 9005-9009). The short time
required to observe the apoptosis (2 h) (Figure 7),
suggests that cell cycle, DNA synthesis, or
5   significant transcription/translation may not be a
pre-requisite for tempo-initiated cell death. It
seems possible that post-translational modification of
existing proteins required for the induction of
apoptosis is regulated by free-radical-mediated
10   protein kinase pathway(s) involving SAPK.
Endogenous sphingolipid metabolites such as
ceramides and sphingosines have been recognized as
lipid mediators of cell growth, differentiation and
apoptosis (Haimovitz-Friedman, A. *et al.*, 1994, *supra*;
15   Obeid, L. M. *et al.*, 1993, *Science* 259, 1769-1771;
Kolesnick, R. and Golde, D. W., 1994, *Cell* 77, 325-
328; Bose, R. *et al.*, 1995, *Cell* 82, 405-414; Jarvis,
W. D. *et al.*, 1994, *Proc. Natl. Acad. Sci. U.S.A.* 91,
73-77; Pushkareva, M. *et al.*, 1995, *Immunol. Today* 16,
20   294-297). Apoptosis has been suggested to be
dependent or independent of ceramide release (Verheij,
C. *et al.*, 1996, *supra*; Westwick, J. K. *et al.* 1995,
supra; Shirakabe, K. *et al.* 1997, *J. Biol. Chem.* 272,
8141-8144; Watts, J. D. *et al.*, 1997, *Proc. Natl.*
25   *Acad. Sci. U.S.A.* 94, 7292-7296), and more recently
ceramide has mbeen shown to interact with mitochondria
leading to generation of reactive oxygen species
(Garcia-Ruis, C. *et al.*, 1997, *J. Biol. Chem.* 272,
11269-11377). In other studies, activation of a
30   family of cysteine proteases with specificity for
aspartic acid residues, also known as caspases, has
been tightly linked with apoptotic cell death, and
this pathway involves the release of cytochrome C from
mitochondria (Liu, x. *et al.*, 1996, *Cell* 86, 147-157;
35   Salvesen, G. S. and Dixit, V. M., 1997, *Cell* 91, 443-

446). Whether ceramide generation in tempo-treated
cells is due to activation of sphingomyelinase and/or
ceramide synthase, or tempo-treatment results in the
activation of caspases are important issues currently
5   under investigation in our laboratory.

What components are upstream of ERK and SAPK in
the tempol- and tempo-initiated signaling,
respectively? Tempol and tempo are uncharged
nitroxides in the physiological pH range and readily
10   cross the cell membrane; however, their concentrations
in subcellular compartments differ. Tempo is
approximately 200 times more lipophilic than tempol
(Kocherginsky, N. *et al.*, 1995, in <u>Nitroxide Spin</u>
<u>Labels, Reactions in Biology and Chemistry</u>, pp. 15-26,
15   CRC Press, Boca Raton, FL), hence tempo would be
expected to accumulate in the cell membrane to a
greater extent than tempol. Thus, having an agent
such as tempo (a stable free radical) localized in the
cell membrane and capable of participating in redox
20   reactions may initiate a signal transduction cascade
distinct from tempol which is more water soluble and
more evenly distributed throughout the cell. Although
both tempol and tempo stimulated Raf-1, ERK1 activity
was increased only in tempol-treated cells. Raf
25   activation was temporal compared to ERK1. Raf-1
activity peaked at 30 min, whereas ERK activity began
to rise at 15 min and continued to rise for at least
upto 120 min. This lack of correlation between the
kinetics of Raf-1 activation and ERK activation has
30   been observed earlier (Kasid, U. *et al.*, 1996, Nature
382, 813-816;Suy, S. *et al.*, 1997, *supra*), and may be
due to multiple effectors, including Raf-1, upstream
of ERK. At present, the significance of Raf-1
activity in a nitroxide-induced response is unclear.

MEK, a known physiological substrate of Raf-1 and
activator of ERK (Dent, P. *et al.*, 1992, *Science* 257,
1404-1407; Howe, L. R. *et al.*, 1992, *Cell* 71, 335-342;
Kyriakis, J. M. *et al.*, 1992, Nature 358, 417-421;
5   Avruch, J. *et al.*, 1994, *Trends Biochem. Sci.* 19, 279-
283; Crews, C. W. *et al.*, 1992, *Science* 258, 478-480;
Marshall, C. J., 1994, *Curr. Opin. Genet. Dev.* 4, 82-
89), and SEK1, a potent activator of SAPK (Derijard,
B. *et al.*, 1995, *supra*; Sanchez, I. *et al.*, 1994,
10   *supra*; Johnson, N. L. *et al.*, 1996, *supra*; Minden, A.
et al., 1994, *supra*; Lin, A. *et al.*, 1995, Science
268, 286-290) are other potential upstream targets.
The regulation of MAPKs including ERK and JNK/SAPK
involves sequential phosphorylations, often initiated
15   at the cell surface by a receptor or non-receptor
protein tyrosine kinase(s). Other reports have
suggested a balance between ERK and SAPK activities as
a determinant of cell survival or cell death (Xia, Z.
et al., 1995, *supra*). Based on a significant
20   increase in protein tyrosine phosphorylation within 15
min after tempo treatment compared to tempol, it is
plausible to speculate the activation of a lipid-
mediated signaling pathway which involves pro-
apoptotic protein tyrosine kinase(s) in tempo-treated
25   cells.

In conclusion, present studies provide evidence
that (a) tempo induces a significant tyrosine
phosphorylation of several as-yet unidentified
proteins as compared to tempol, (b) tempol and tempo
30   stimulate tyrosine phosphorylation and activity of
Raf-1 protein kinase, (c) tempol stimulates MAPK (ERK)
activity, wheras tempo is a potent inducer of SAPK
phosphorylation and activity, (d) tempo, but not
tempol, induces apoptotic cell death, and (e) tempo-
35   induced cell death could be associated with ceramide

genertion in MDA-MB231 cells.   Our findings imply that
in the absence of an environmental oxidative stress,
such as that induced by ionizing radiation, nitroxides
tempol and tempo stimulate distinct signal

5   transduction pathways, perhaps triggered by secondary
radicals associated with cellular metabolism and
differentially regulated by early events, such as the
control of protein tyrosine phosphorylation and
generation of ceramide.

10   The MAP kinase pathway is a widely used signal
trnsduction mechanism that initiates proliferation.
Hyperexpresion of MAP kinase has been localized to
malignant breast epithelium and metastatic cells of
patients with breast cancer (Sivaraman, V. S. et al.,

15   1997, *J. Clin. Invest.* 99, 1478-1483).   Identification
of compounds activating a cell death pathway(s) should
then lead to their rational use in cancer therapy.
The finding that tempo induces apoptosis in different
cell types warrants further study.   It is most

20   interesting that an agent that exerts antioxidant
activity can also induce cytotoxicity by apoptosis.
Should there be a differential induction of apoptosis
in human tumor versus normal cells, the use of tempo
may have clinical utility.   Studies are presently

25   under way in our laboratory to explore this
possibility.

30

35

What is claimed is:

1. A method for inducing cell death comprising

5　administering to said cell a composition comprising
tempo or a functional derivative of tempo in an amount
sufficient to induce death of said cell.

2. The method according to claim 1 wherein said

10　cell is a cancer cell or a diseased cell.

3. The method according to claim 1 wherein said
tempo is administered in combination with other
anticancer treatments.

15

4. A pharmaceutical composition for reducing
tumors, said pharmaceutical composition comprising
tempo or a functional derivative of tempo in a
pharmaceutically effective amount in a pharmaceutical

20　diluent.

5. The pharmaceutical composition of claim 4
wherein said tumor is of prostate, breast, ovary, head
and neck, kidney, lungs, bone, brain, pancreas, and

25　liver.

6. A method for activation of the caspase
signaling cascade in a cell comprising administering
to said cell a composition comprising tempo or a

30　functional derivative of tempo in an amount sufficient
to activate the caspase cascade.

7. The method according to claim 6 wherein said
caspase is caspase-3.

8. A method for activation of the SAPK signaling
cascade in a cell comprising administering to said
cell a composition comprising tempo or a functional
derivative of tempo in an amount sufficient to
activate the SAPK cascade.

9. A method for activating an apoptotic signaling
cascade in a cell comprising administering to said
cell a composition comprising tempo of a functional
derivative of tempo in an amount sufficient to
activate said cascade.

10. The method according to claim 1 wherein said
tempo is conjugated to a carrier.

11. A composition for inducing cell death
comprising tempo.

12. The composition of claim 11 wherein said
tempo is conjugated to a carrier.

13. The composition of claim 12 wherein said
carrier is chosen from the group consisting of:
ligand, growth factor, cytokine and liposome.
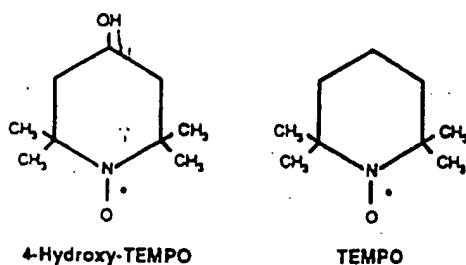
FIG. 1. Chemical structures of nitroxide compounds tempol
(*4-hydroxy-tempo*) and tempo.
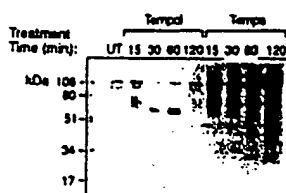
FIG. 2. Effects of tempol and tempo on protein tyrosine phos-
phorylation.

A

B
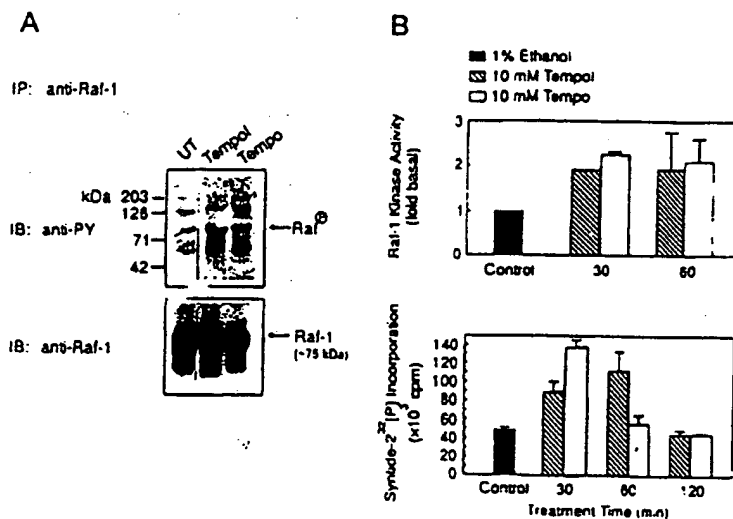
FIG. 3. Tempol and tempo stimulate tyrosine phosphorylation and enzymatic activity of Raf-1 protein kinase in vivo.
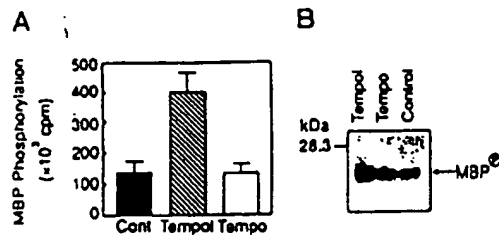
Fig. 4. Tempol stimulates ERK1 activity.

Fig. 6. Effects of tempol and tempo on cell viability.
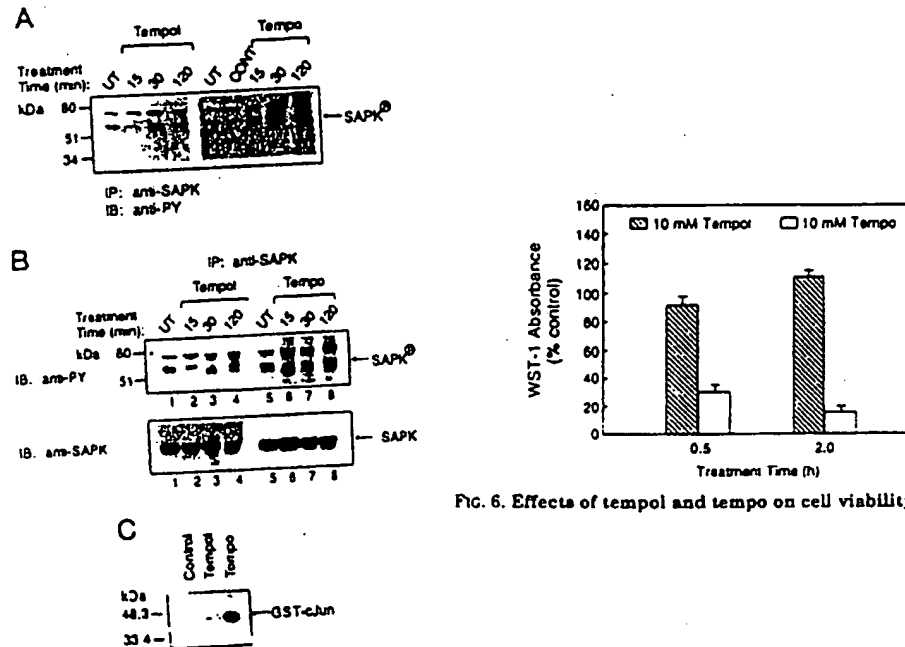
Fig. 5. Tempo stimulates tyrosine phosphorylation and activity of SAPK.

Fig. 8. Ceramide production in tempo-treated MDA-MB 231 cells.
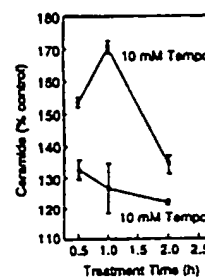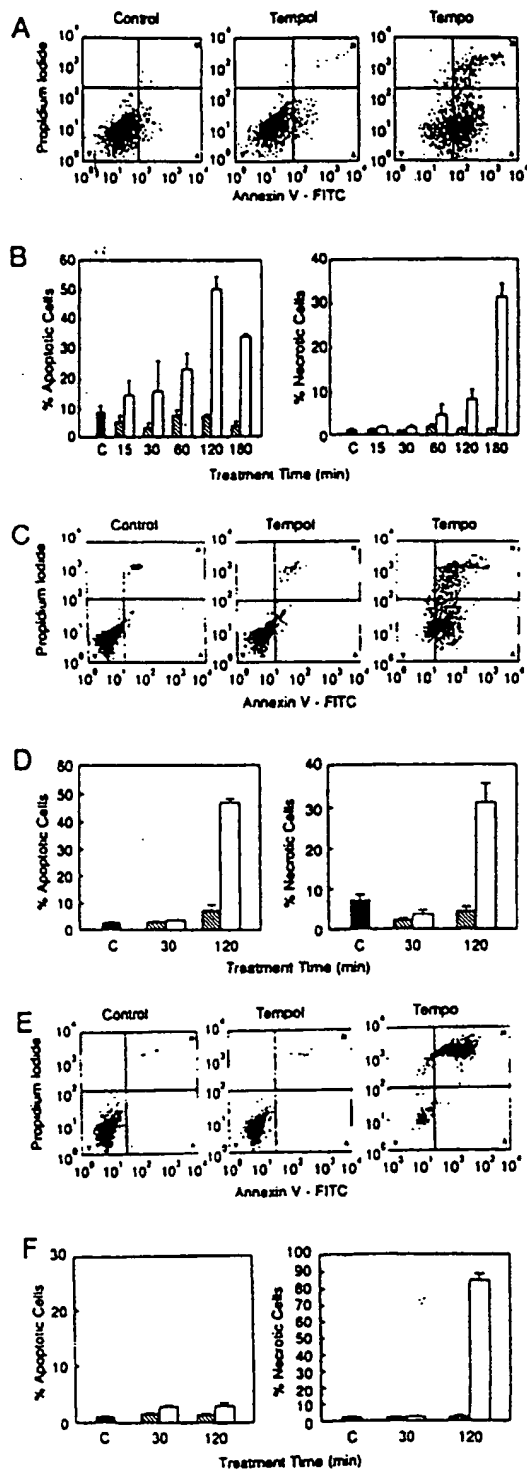
FIG. 7. Tempo induces apoptotic cell death.

A

B

C

D
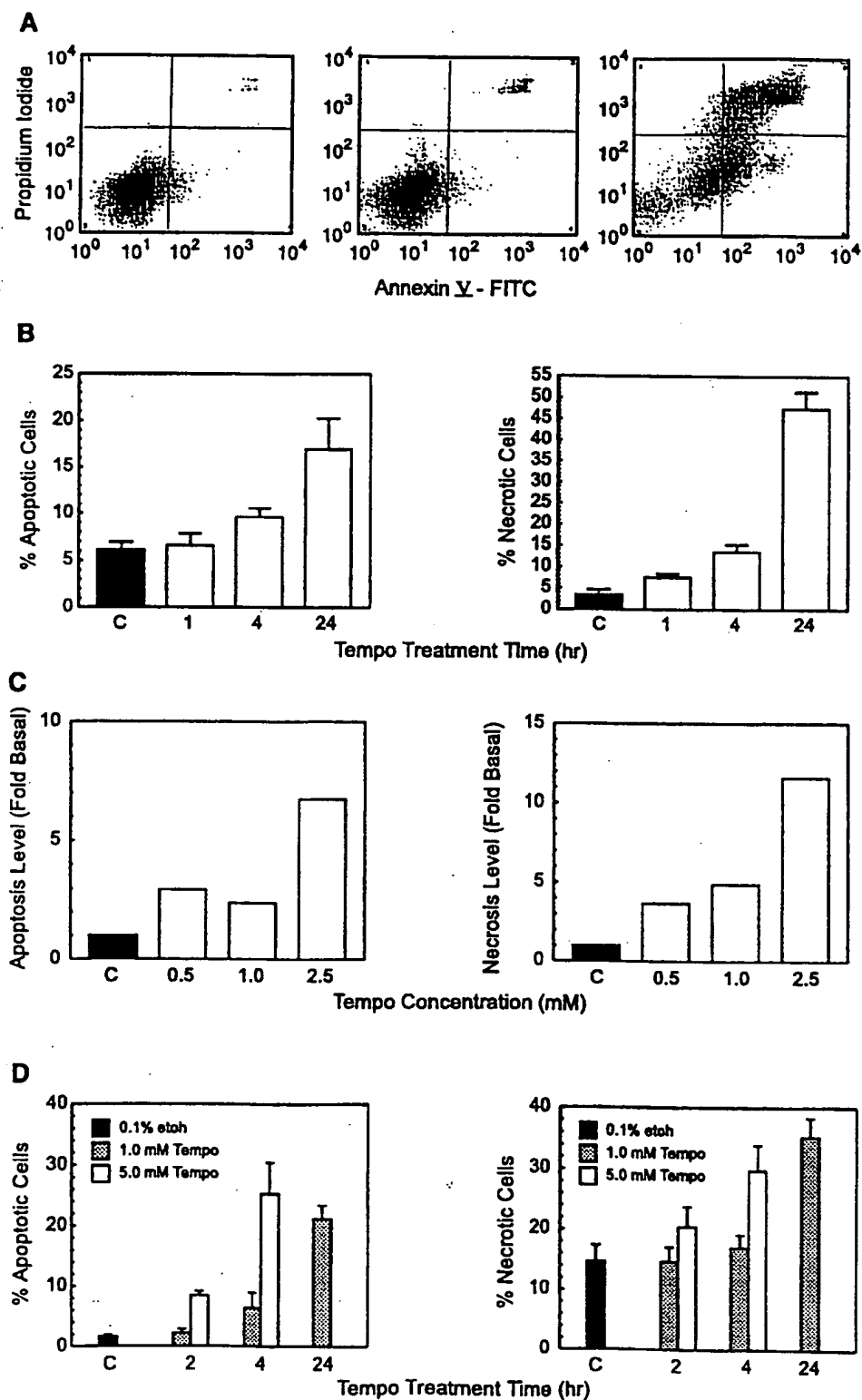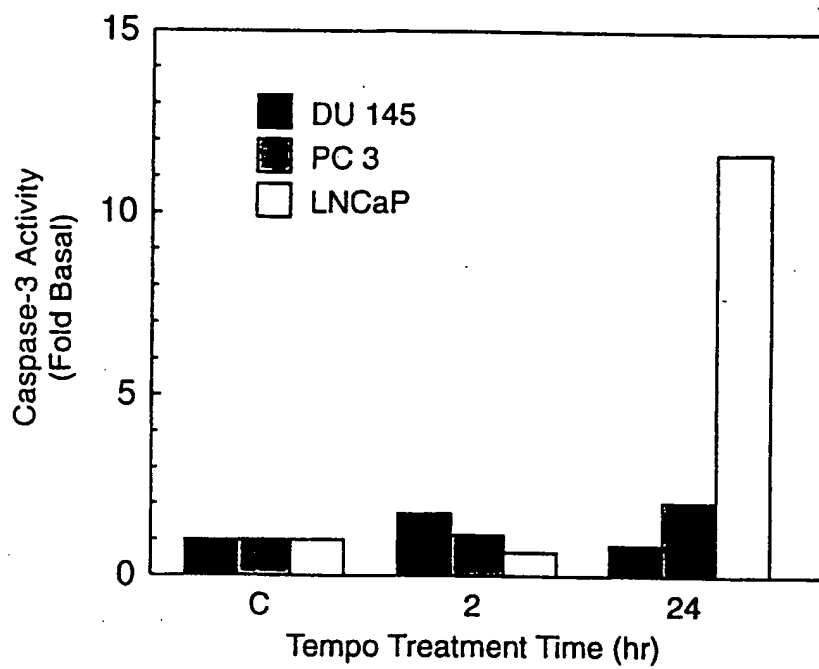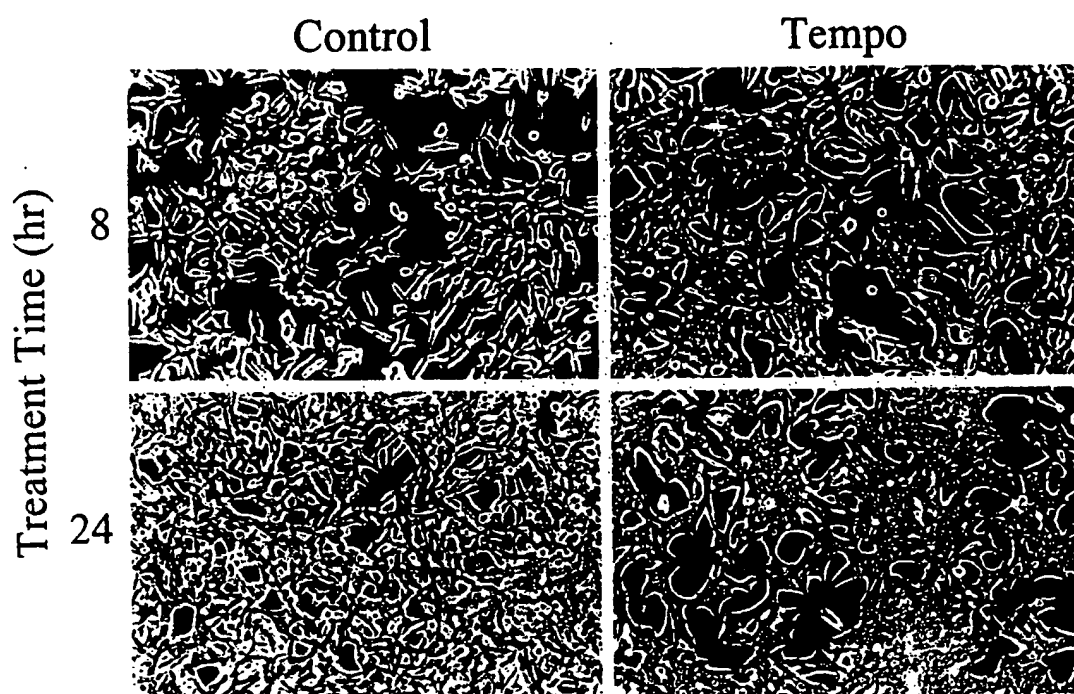
FIGURE 9

FIGURE 10

FIGURE 11
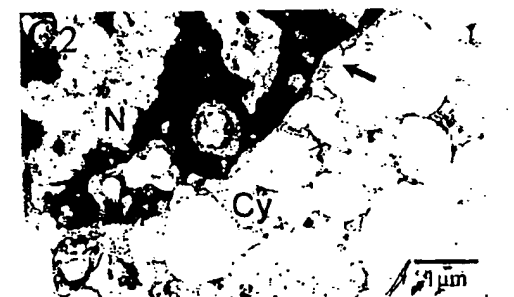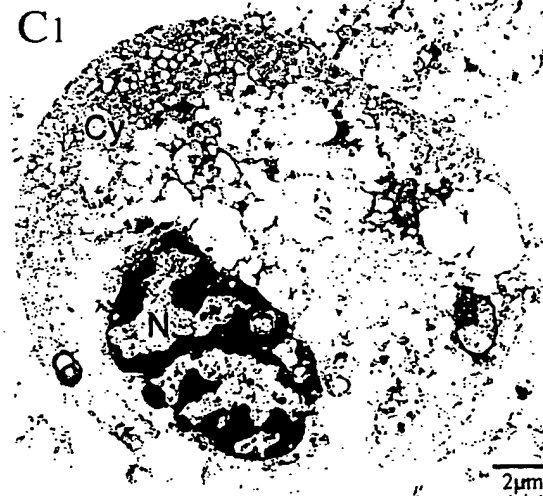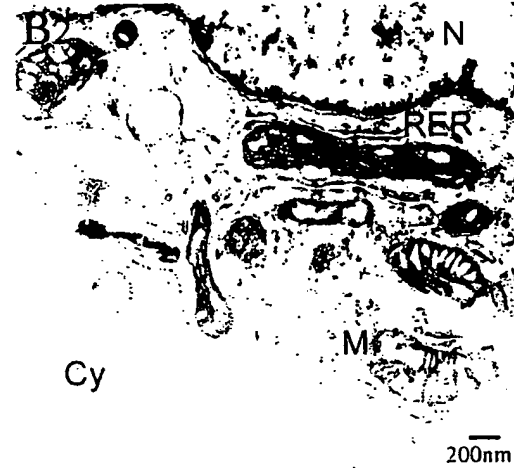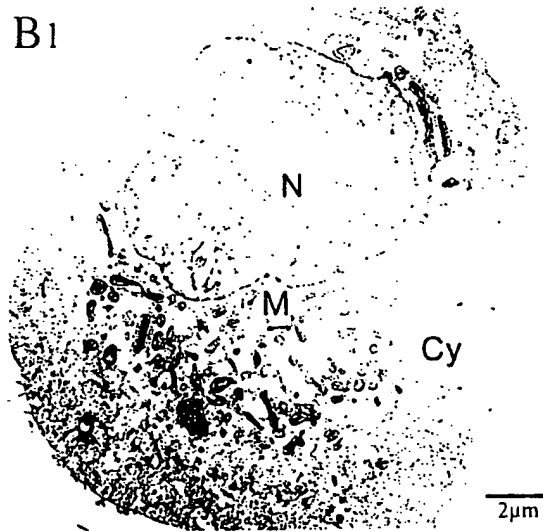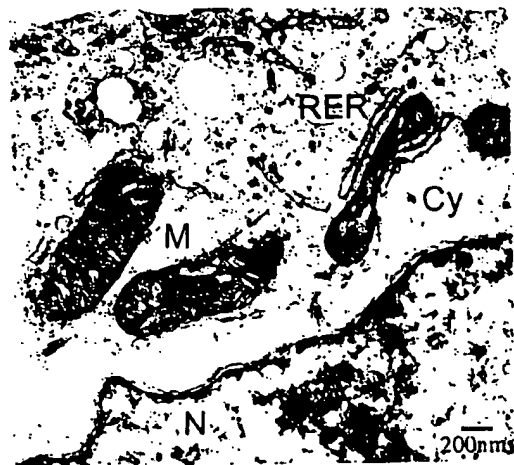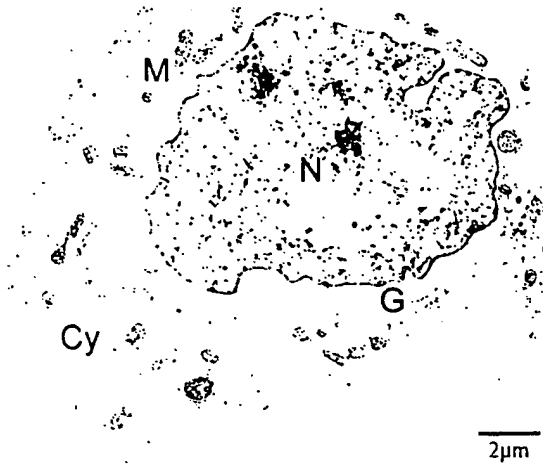
FIGURE

A.
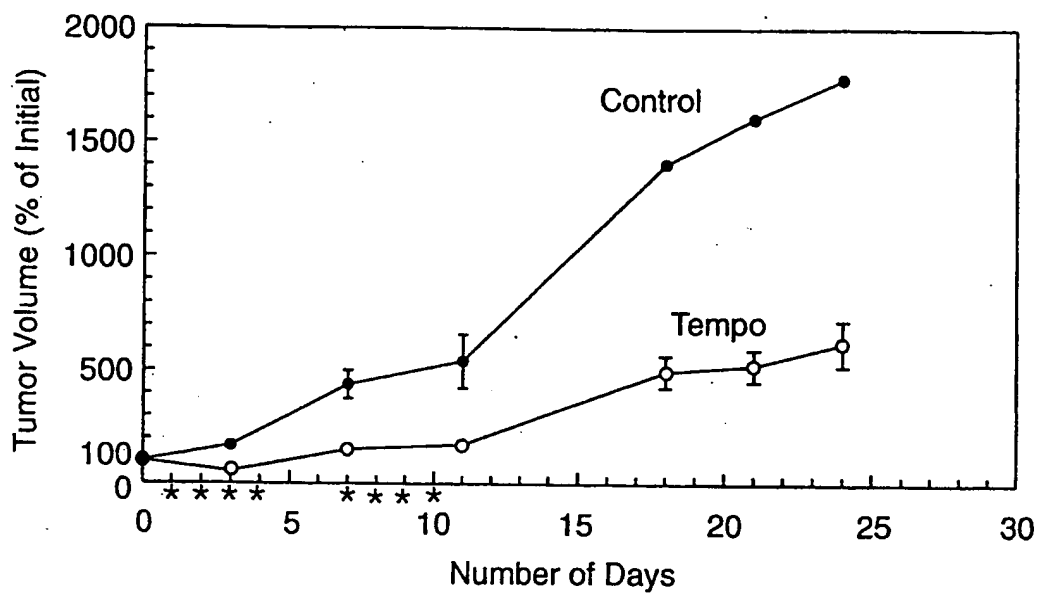
B.

Control   Tempo

FIGURE 13